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14. ABSTRACT Our hypothesis is that reversible alterations in histones are a determining factor for low FANCD2 expression in ovarian surface epithelial (OSE) cells in women with a familial risk for ovarian cancer, and that cells with reduced FANCD2 levels are hypersensitive to the genotoxic effects of estrogen, therefore predisposing OSE to malignant transformation. During this year we have screened a large number of normal (no familial risk), high-risk (with familial history of this disease), and ovarian cancer cell lines, and determined levels of FANCD2 protein and mRNA. In the first set of experiments, aimed at determining whether histone modifications (i.e. acetylation and/or methylation) affect FANCD2 levels, we established that Trichostatin A (TSA;10nM for 24 hours) corrects FANCD2 levels.In the second set of experiments, aimed at establishing whether the estrogen metabolite 4-OHE2 is genotoxic for cells with low FANCD2 levels we: 1) identified the minimal concentration of 4-OHE2 that is associated with DNA damage in human and murine OSE, and; 2) found that OSE cultures that express low FANCD2 exhibit significantly increased DNA damage after exposure to 50 uM 4-OHE2, in comparison with OSE cultures with normal levels of FANCD2 protein expression.

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High risk OSE, FANCD2, HDACi, 4-OHE2, Comet assay, DNA damage

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2013 Annual Progress Report

INTRODUCTION

Central to our application is our previous finding of decreased expression of FANCD2, a key protein in Fanconi (FA)/BRCA DNA repair pathway) in cultured ovarian surface epithelial (OSE) cells from women with a family history of ovarian cancer and women with ovarian cancer. We previously demonstrated that increasing FANCD2 expression in these cells improved their genetic stability, consistent with a causal relation between higher FANCD2, improved genetic integrity and decreased risk of ovarian cancer. The mechanism by which reduced FANCD2 may render OSE sensitive to neoplastic transformation is not known, but evidence suggests that such OSE cells may be sensitive to toxic effects of estrogen on genomic material and that loss of FANCD2 limits DNA repair potential in these cells. **The overall** objectives of the proposal are thus to delineate the molecular and genetic mechanisms underlying (1) reduced FANCD2 expression and (2) the etiology of cancer in FANCD2-deficient women. Our hypothesis is that reversible histone alterations (methylation and/or acetylation) are a determining factor for low FANCD2 expression in high risk OSE and that cells with reduced FANCD2 levels are hypersensitive to the genotoxic effects of estrogen, therefore predisposing OSE to malignant transformation. Two specific aims are being pursued to test this.

Specific Aims:
I. Determine the epigenetic mechanisms of FANCD2 downregulation in the OSE of women at high risk for ovarian cancer, and to examine how normalizing FANCD2 expression might stabilize genome in high-risk women.

II. Evaluate estrogen-induced genotoxicity as an etiological agent in ovarian cancer in women with alterations in the FA/BRCA Pathway.

BODY – Progress Summary

Specific Aim 1: Determine the epigenetic mechanism of reduced *FANCD2* expression in OSE cells from high-risk women, and whether treatment with histone deacetylase inhibitors (HDACi) improves survival of FANCD2-low cells and whether normalized *FANCD2* expression suppresses cross-linker-induced chromosomal breakage and cell toxicity.

1. We have amended our existing IRB approved protocols OHSU IRB 921 and OHSU 3485 to include Department of Defense sponsored research. Both IRB amended and approved protocols have subsequently been accepted by the DOD Research Protection Office approval.
2. Initially we cultured 100 human OSE samples from our Biorepository. Successful cultures were obtained from 78 human OSE cell lines. FANCD2 protein levels were determined using Western blot method in our laboratory, with Santa Cruz FANCD2 antibody. We have screened 17 normal OSE, 26 high-risk (HR) OSE and 35 ovarian cancer cell lines for FANCD2 protein and mRNA levels. The reduced levels of both FANCD2 protein and mRNA suggested epigenetic mechanism of FANCD2 regulation. We have previously shown that FANCD2 promoter is not methylated (1). However DNA histone modification may also regulate gene expression (2); therefore, cells with reduced FANCD2 levels were treated with trichostatin-A (TSA) at 10 nM concentration, 5-aza-2'-deoxycytidine (5-aza) 0.5 uM and combination of TSA and 5-aza and several time points. The changes in FANCD2 protein levels were evaluated by densitometry. The pattern of response to TSA and 5-aza was variable (Figure 1). Additional work is underway to clearly identify the merits of future efforts.

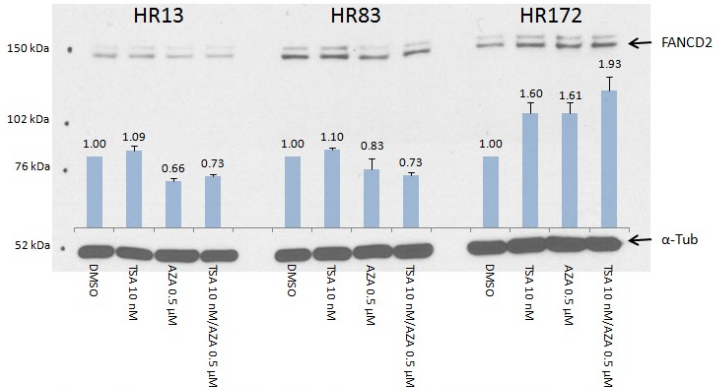

Figure 1. Three primary high risk cell lines that were treated with 10nM Trichostatin A (TSA) and 0.5μM 5-Aza-2'-deoxycytidine (AZA). Top band located at approximately 155 kDa is the FANCD2 protein. Lower band at approximately 50 kDa is loading control alpha-tubulin. Graph represents adjusted density of band as compared to DMSO using Image J by NIH.

Specific aim 2: We aim to evaluate estrogen-induced genotoxicity as an etiological agent in ovarian cancer in women and mice with alterations in *FANCD2* expression. (A) Determine if reduced *FANCD2* expression renders OSE cells sensitive to estrogen-induced chromosomal instability.

Comet assay was used to assess genotoxicity of the estrogen metabolite 4-hydroxiestradiol (4-OHE2) in vitro, in 20 human OSE cell lines, with various FANCD2 protein levels and 2 mouse cell lines (wild type and Fancd2 -/- mouse). Collected cells were brought to $1.5x10^5$ cell/mL with PBS. Comet assay was performed using Trevigen Comet Assay reagents for Single Cell gel Electrophoresis. Cells were suspended in low melting point agarose, plated on slides and allowed to harden for 10 minutes at +4^o C. Slides were then washed, incubated in 70% ethanol and allowed to dry overnight at room temperature. Samples were stained with SYBR Green and viewed using fluorescent microscope. Images were analyzed by scoring 100 cells per condition and determining percent DNA in tail using CometScore (TriTek Corp, Sumerduck, VA, USA).Primary OSE cells were cultured in Phenol Red-Free DMEM/RPMI (GIBCO, Life Technologies) with 10% Charcoal/Dextran treated FBS (ThermoScientific) in6 well plates with 2 x 10% cells per well. Untreated cells were included as negative controls. Cells treated with 50 uM Camptothecin were used as positive controls. Experimental cells, to test the effects of the estrogen metabolite were exposed for 2 hours with 50 uM and 75 uM 4-OHE2 at 37 ^{o}C and then harvested by trypsinization. The data was analyzed using percent DNA in tail (CometScore). A non-parametric test was used to assess the differences in DNA damage between 11 cell lines with reduced FANCD2 levels and 8 FANCD2 positive cell lines. Only 50 uM 4-OHE2 showed significant differences in this assay (Table 1).

Table 1.

	Low (n=11)	Positive (n=8)	T test P-value	P-value *
Control, Mean(SD)	4.43 (0.96)	4.66 (0.15)	0.4880	0.1731
Camp, Mean(SD)	80.49 (6.93)	75.27 (9.37)	0.1866	0.2006
50*u*m, Mean(SD)	7.80 (2.68)	13.53 (7.11)	0.0445	0.0093
70*u*m, Mean(SD)	18.60 (12.22)	33.33 (16.03)	0.0358	0.1074

* Wilcoxon- Mann-Whitney test (Nonparametric test)

The DNA damage response to 4-OHE2 in low FANCD2 expressing cells is not uniformely increased as expected. One explanation may be in that that the Comet Assay detects DNA damage in the form of DNA single strand breaks but also cross-links which move slower within Comet assay and are therefore not as readily detected as single strands DNA products(3, 4); therefore, an alternative technique to detect DNA damage may be utilized. An approach used in this lab is cytogenetic analysis which is able to specifically detect chromosomal breaks and radials.

The two murine cell lines showed increased DNA tails with exposure to 25 microM 4OHE2. This provides an initial rationale to develop an in vivo murine model, which currently has IACUC approval to perform the proposed protocols.

Key Research Accomplishments:

1. We identified the minimal concentration of 4-OHE2 that is associated with DNA damage in human and murine OSE.
2. FANCD2 low OSEs exhibit significantly increased DNA damage after exposure to 50 uM 4-OHE2 in comparison with OSE with normal FANCD2 protein expression.
3. We identified that TSA corrects the FANCD2 level in some high-risk and ovarian cancer OSE at concentration of 10 nM after 24 hours exposure.

Reportable Outcomes:

Publications: None
Meeting abstracts: None
Cell lines developed: None
Tissue and serum repositories: None
Informatics and databases: None
Animal models: None

Funding applied for based on this award:
1.Intramural Mission Award, Department of Ob/Gyn OHSU June 2013 (one year).
2.DOD Translational Leverage Award 2014, submitted based in part on the results related to FANCD2 expression.

Employment or research opportunities applied for or received based on this award: 1.Melissa Kellar, B.Sci.
2.Dr. Jay Wright, PhD, funding and personnel change.

Conclusion: We have met our initial expectations of completing suggested experiments timely. Our results support the notion that (1) TSA may correct FANCD2 expression in ovarian epithelial cells characterized by low FANCD2 expression and (2) estrogen byproduct 4-OHE2 induces cDNA damage in human and mouse cells with low FANCD2.

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Appendices:
None (table and Figure are given in the text of the report).